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Development of High Sensitivity Capillary Electrophoresis Fluorescence System for the Detection of Biological Molecules *(Final Report)*

Norman J. Dovichi
University of Alberta

Scientific Authority:
C. Boulet
Defence Research Establishment Suffield

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Development of High Sensitivity Capillary Electrophoresis Fluorescence System for the Detection of Biological Molecules

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Norman J. Dovichi

Department of Chemistry

University of Alberta

Introduction

Chemical and biological weapons fall into three classes. The first class consists of small, synthetic molecules, which are considered as the classic chemical warfare agent. The second class of agents consists of biological organisms, including bacteria and viruses, which are the classic biological warfare agents. The third class of compounds consists of toxic proteins and peptides produced by biological organisms. This contract considered the analysis and detection of these mid-spectrum agents.

There are two basic strategies for the detection and identification of the mid-spectrum agents. Antibody-based immunoassays form one class of assays. These assays rely on the highly specific reaction between the antibody and its antigen. The specificity of this reaction is both its strength and weakness. There is relatively little cross-reactivity of the reagent with other molecules, so that false-alarms are expected to be relatively rare. However, immunoassays suffer from two problems. First, a positive signal is observed only if an antibody is used that recognizes the antigen. Novel or unexpected mid-range

agents are not recognized by the immunoassay. Second, the large number of antibodies needed to screen most possible agents presents a logistical hurdle in the field.

The second strategy for mid-agent analysis is based on chemical methods. Here, the agent is analyzed by an electrophoretic or mass-spectroscopic method. Mass spectrometry is a rapid method that provides precise and accurate molecular weight information on the agent. However, mass spectrometry is relatively insensitive, requiring nanomolar or higher concentration samples. In contrast, capillary electrophoresis when coupled with laser-induced fluorescence can provide exquisite sensitivity.

In the first part of this project, we investigated simple methods of fluorescently labeling proteins and peptides, with subsequent separation and analysis by capillary electrophoresis with laser-induced fluorescence. The labeling reagents included tetramethyl rhodamine succinimidyl ester, 3-(p-carboxybenzoyl)quinoline-2-carboxyaldehyde (CBQ), and 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ). The latter reagent provided the lowest background and cleanest electropherograms. We then constructed a prototype instrument for the analysis of the labeled proteins. This instrument produced detection limits of 150 molecules of fluorescein injected onto the column, which represents the state-of-the-art in fluorescence detection. The system was delivered to DRES and DRES personnel were trained in its use.

The instrument and labeling technology were used to evaluate samples generated during a field trial at Dugway. The experimental results revealed that the protein sample dispersed during the field trial had undergone significant degradation during its handling. This degradation quite likely occurred before the protein was dispersed; the protein solution was an ideal bacterial growth medium. However, the sample, once collected, was

not protected from further degradation. The field-trial pointed out the care required for sample preservation before analysis.

Literature review

Capillary electrophoresis is a powerful separation tool for proteins. However, conventional ultraviolet absorbance detection produces poor concentration detection limits, which prohibits the use of capillary electrophoresis for trace-level analysis. Laser-induced fluorescence can provide highly sensitive detection of very low concentrations of native and derivatized proteins (1-12). Native fluorescence detection usually requires the use of expensive and temperamental lasers that operate in the ultraviolet portion of the spectrum. Derivatized proteins can be detected with relatively inexpensive air-cooled argon ion or helium-neon lasers; however, derivatization chemistry can suffer not only from interference due to excess derivatizing reagent but also from complex electropherograms generated by multiple reaction products due to incomplete labeling of all possible sites on the protein (13-14).

Fluorogenic reagents have proven useful for protein analysis (15). These reagents are not fluorescent until they react with a primary amine; they generate low background signals compared to traditional fluorescent derivatizing reagents. We have exploited 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ) to generate highly fluorescent proteins that are separated efficiently when used with a submicellar buffer system, without noticeable band-broadening due to multiple labeling (11). We reported the successful labeling and analysis of a 10^{-11} M conalbumin by this method.

Unfortunately, most labeling chemistry is cumbersome and difficult to automate. In this paper, we report the automated reaction between protein and FQ in the capillary as

a form of electrophoretically-mediated microanalysis (EMMA) (16-20). In EMMA, reagents are introduced in order of increasing mobility. Under the influence of an electric field, the reagents mix and react to form a product. The time during which the reagent zones overlap determines the reaction time, which can be controlled by stopping the electric field after the reagent zones mix. After the reaction is complete, the product is separated from the reagent using zone electrophoresis. By performing the reaction at 65 °C rather than room temperature, we obtain a tenfold improvement in detection limit for conalbumin and reduce the total analysis time from 35 to 5 minutes. Picomolar concentration and zeptomole amounts of protein are analyzed by EMMA-CE-LIF.

Research results

The proposal had three research themes. The first dealt with improved detection sensitivity. The second dealt with improved identification through use of sieving media for the electrophoretic separation. The third dealt with on-column reaction chemistry to automatically label proteins before electrophoretic separation and fluorescence detection. All three topics were successfully completed and are described in three different publication, two of which appeared in *Analytical Chemistry* and the third in *Electrophoresis*. Copies of these papers are attached to this report; details of the experiment and results are found there. This document summarizes our results.

The first paper, "Picomolar assay of native proteins by capillary electrophoresis - precolumn labeling, sub-micellar separation, and laser induced fluorescence detection" D.M. Pinto, E.A. Arriaga, D. Craig, J. Angelova, N. Sharma, H. Ahmadzadeh, N.J. Dovichi, C.A. Boulet, *Analytical Chemistry* 69, 3015-3021 (1997), reported our assay method using FQ to label proteins. We reported a method for the assay of proteins at concentrations lower than 10^{-10} M with as little as 200 amol of protein. High sensitivity was accomplished by derivatizing the ε-amino group of the protein's lysine residues with

the fluorogenic dye 5-furoylquinoline-3-carboxaldehyde (FQ) and use of a sheath-flow cuvette fluorescence detector. Most proteins have a large number of lysine residues, therefore, a large number of fluorescent molecules can be attached to each protein molecule. In general, pre-column labeling improves sensitivity but degrades resolution due to the inhomogeneity of the reaction products from multiple labeling. However, we demonstrated that, through careful manipulation of the separation and reaction conditions, high sensitivity can be obtained without excessive loss in separation efficiency. Over 190,000 theoretical plates were obtained for fluorescently labeled ovalbumin.

The second paper, "SDS-capillary electrophoresis of proteins in a sieving matrix utilizing two-spectral channel laser-induced fluorescence detection" D.B. Craig, R. Polakowski, J.C.Y. Wong, H. Ahmadzadeh, C. Stathakis and N.J. Dovichi, *Electrophoresis* 19, 2175-2179 (1998), reported the use of sieving media for the separation of labeled proteins. We used a 7.5% dextran solution for the separation. This media gave a separation of proteins that was size-dependent. However, we found that accurate size-measurement was difficult without the simultaneous analysis of size-standards. Using our NSERC research grant funds, we constructed a two-color instrument that would simultaneously measure fluorescence from analyte and from a set of size standards. Different dyes were used to label the standard and sample proteins. A two-spectral channel detector resolved fluorescence from the sample and standards. Comparison of the migration time of the sample and standards permitted the precise determination of molecular weight, irrespective of variations in run-to-run migration times.

The third paper, "Picomolar analysis of proteins using electrophoretically mediated microanalysis and capillary electrophoresis with laser induced fluorescence

detection" I.H. Lee, D. Pinto, E.A. Arriaga, Z. Zhang, N.J. Dovichi, *Analytical Chemistry* in press, dealt with the on-column labeling and analysis of proteins with a fluorogenic reagent. Labeling was followed by capillary zone electrophoresis separation and post-column detection based on laser-induced fluorescence. The method provided a concentration detection limit (3 σ) of 3×10^{-13} M for conalbumin. The method provided separation efficiency of 400,000 theoretical plates. Protein extract from a human colon adenocarcinoma cell line generated a dozen major components and many minor components in a 12-minute separation; the protein extract from 2.5 cells was used for this analysis. When compared to UV absorbance detection, the EMMA method provided 7,000,000-fold improvement in detection limit and a 13-fold improvement in efficiency.

The last research result consists of Devanand Pinto's PhD thesis. This contract, and its predecessor, supported much of Dev's thesis research. It is fitting that a bound copy of his thesis accompanies this final report.

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Picomolar Assay of Native Proteins by Capillary Electrophoresis Precolumn Labeling, Submicellar Separation, and Laser-Induced Fluorescence Detection

Devanand M. Pinto, Edgar A. Arriaga, Doug Craig, Jordanka Angelova, Neepun Sharma, Hossein Ahmadzadeh, and Norman J. Dovichi

Department of Chemistry, University of Alberta, Edmonton, Canada, T6G 2G2

Camille A. Boulet

Defense Research Establishment Suffield, Suffield, Canada, P.O. Box 4000, Medicine Hat, Alberta, Canada, T1A 8K6

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Devanand M. Pinto, Edgar A. Arriaga, Doug Craig, Jordanka Angelova, Neepun Sharma, Hossein Ahmadzadeh, and Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Canada, T6G 2G2

Camille A. Boulet

Defense Research Establishment Suffield, Suffield, Canada, P.O. Box 4000, Medicine Hat, Alberta, Canada, T1A 8K6

We report a method for the assay of proteins at concentrations lower than 10^{-10} M with as little as 200 amol of protein. High sensitivity is accomplished by derivatizing the ϵ-amino group of the protein's lysine residues with the fluorogenic dye 5-furoylquinoline-3-carboxaldehyde and use of a sheath flow cuvette fluorescence detector. Most proteins have a large number of lysine residues; therefore, a large number of fluorescent molecules can be attached to each protein molecule. In general, precolumn labeling improves sensitivity but degrades resolution due to the inhomogeneity of the reaction products from multiple labeling. However, we demonstrate that, through careful manipulation of the separation and reaction conditions, high sensitivity can be obtained without excessive loss in separation efficiency. Over 190 000 theoretical plates are obtained for fluorescently labeled ovalbumin.

The high separation efficiency of capillary electrophoresis is valuable for the analysis of proteins.[1,2] Unfortunately, concentration detection limits of UV absorbance in capillary electrophoresis often fall in the micromolar range. In biological samples, where nanomolar protein concentration is common, improved sensitivity is essential.

Techniques developed to improve the concentration detection limit include field amplification,[3,4] capillary isotachophoresis,[5] capillary isoelectric focusing,[6] on-column concentration using reversed phase adsorbents,[7] and on-column concentration using a small-pore plug.[8] These methods improve the concentration detection limit by ~2 orders of magnitude.

There have also been significant advances in the design of sensitive detection schemes including laser-induced fluorescence (LIF), thermooptical absorbance (TOA), and mass spectrometry.[9−15] Native fluorescence detection makes use of a UV laser operating near 250 nm to excite the protein's aromatic amino acids, with tryptophan being the most important. When combined with on-column stacking, native fluorescence has generated subnanomolar concentration detection limits for bovine serum albumin (BSA) and conalbumin.[9] Tryptophan is a relatively rare amino acid, and not all proteins will generate useful native fluorescence signals. Also, lasers that operate in the ultraviolet region tend to be expensive and somewhat temperamental in operation.

On the other hand, lasers that operate in the visible portion of the spectrum tend to be rugged and relatively inexpensive. We recently reported the use of low-power lasers and sheath flow cuvette technology to achieve a detection limit of six molecules of a rhodamine dye and single molecules of the highly fluorescent protein B-phycoerythrin.[16,17] To extend this technology to the analysis of biomolecules, we have developed fluorescent labeling schemes for the analysis of DNA, peptides, sugars, and amino acids.[18−21]

Fluorescent labels, such as fluorescein isothiocyanate (FITC) and fluorescamine have been used for precolumn labeling of peptides.[22,23] Reactions are usually performed with proteins at

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millimolar concentrations followed by dilution by several orders of magnitude before analysis. It is much more difficult to fluorescently label dilute solutions of proteins. Most fluorescent labeling reagents undergo hydrolysis in aqueous environments. The presence of these competing reactions necessitates the use of high concentrations of labeling reagents, typically in the millimolar range, in order to obtain efficient labeling. Unfortunately, the high background signal from excess fluorescent reagent and hydrolysis products often swamps the fluorescence signal from dilute protein solutions. More problematic, impurities present at the part-per-million level in the fluorescent labeling reagent create a forest of background peaks that inevitably interferes with the protein signal. An attractive alternative to aqueous-phase labeling is solid-phase labeling. We recently demonstrated that, in the case of a peptide, the use of solid-phase labeling avoids some of the drawbacks of precolumn labeling.[18] Several novel applications of solid-phase labeling using immobilized reagents have been developed. Szule and Krull, who have performed much of this work, recently published a review of the topic.[24] While attractive for the analysis of small molecules and peptides, solid-phase labeling of proteins has not been demonstrated.

An attractive alternative for the labeling of dilute proteins is the use of fluorogenic reagents.[25] These nonfluorescent reagents form a fluorescent product when they react with primary amines. Unfortunately, fluorogenic reagents also react slowly to form secondary fluorescent products.[21] Nevertheless, the use of a fluorogenic derivatizing reagent in place of a fluorescent reagent reduces the background by orders of magnitude.

Precolumn labeling of proteins can degrade separation efficiency due to the heterogeneity of the reaction products formed from multiple labeling. If amino groups are targeted, then both the N-terminus and lysine ϵ-amino groups can be labeled. For a protein with n free amino groups (N-terminus plus lysine residues), there are $2^n - 1$ possible products.[26] For ovalbumin, a glycoprotein that has 20 lysine residues plus an N-terminus, there are 1 048 575 possible reaction products! If each product has a different mobility, the resulting electropherogram is far too complex to be useful. In practice, multiple labeling results in broad peak profiles and low plate counts.[9,24]

We reported a method for eliminating multiple labeling in peptides based on the use of phenyl isothiocyanate to block lysine's ϵ-amino group.[26] Unfortunately, the reaction chemistry is cumbersome. Postcolumn labeling is another method designed to take advantage of the sensitivity of LIF detection.[27] However, mixing of the capillary eluent and the labeling reagent often results in degradation of separation efficiency.

In this article, we reconsider precolumn labeling for trace analysis of proteins. Through careful chemistry, we demonstrate labeling reactions with proteins at picomolar concentration. We also demonstrate that optimization of reaction and separation conditions minimizes band broadening due to multiple labeling. The separation efficiencies are comparable to those of CZE of nonderivatized proteins.

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EXPERIMENTAL SECTION

Instrumentation. Experiments were carried out using a Waters Quanta 4000 CE with a UV absorbance detector, a Beckman 5010 P/ACE with an on-column LIF detector, or an in-house-constructed CE-LIF instrument with a detector based on a sheath flow cuvette as previously described.[28,29] Unless otherwise stated, 50 cm × 50 μm (i.d.) bare fused-silica capillaries (Polymicro Technologies, Phoenix, AZ) were used. The 488-nm line from an argon ion laser and a 615DF45 interference filter were used in the Beckman CE. Absorbance measurements were at 214 nm.

The in-house instrument used a 0–30-kV dc power supply (CZE1000, Spellman, Plainview, NY). For the sheath flow cuvette detector, excitation was provided by the 488-nm line of an argon ion laser operated at 12 mW (Model 2211-15SL, Uniphase, San Jose, CA or Model Innova 90-4, Coherent, Mountain View, CA), which was focused ~30 μm from the tip of the capillary with a 6.3× objective (Melles Griot, Nepean, ON, Canada). Fluorescence was collected by a 60×, 0.7 NA microscope objective (MO-0060LWD, Universe Kokagu, Oyster Bay, NY) filtered with a spatial filter to remove stray light and either a 615DF45 or a 630DF30 bandpass filter (Omega Optical, Brattleboro, VT) to remove scattered light, and imaged onto a photomultiplier tube (R1477, Hamamatsu, Middlesex, NJ) biased at either 1.0 or 1.2 kV. The photocurrent was passed through a current to voltage converter and a low-pass filter (RC = 47 ms) and then digitized with a 16-bit data acquisition board (NB-MIO16XH-18, National Instruments, Austin, TX) connected to a Macintosh computer.

The critical micellar concentration (cmc) of sodium dodecyl sulfate (SDS) in borax buffer was determined using a Model 1650-A impedance bridge from General Radio Co. (Boston, MA). The dc conductivity of the solution measured as SDS was added to 2.5 mM borax, pH 9.5 at 24 °C. The measurement was made for SDS concentrations ranging from 0 to 30 mM and the cmc was determined graphically.

Reagents. Sodium tetraborate (borax) and SDS were purchased from BDH (Toronto, ON, Canada). Enzyme grade 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) was purchased from Fisher Scientific (Fair Lawn, NJ). 2-Mercaptoethanol was purchased from Caledon Laboratories Ltd. (Edmonton, AB, Canada). Tricine was purchased from Sigma (St. Louis, MO). For most CZE experiments, the running and sheath flow buffers were 2.5 mM borax and 5 mM SDS. For some CZE experiments, 25 mM tricine (pH 8.0) was used as the running buffer. The running and sheath flow buffer for CGE experiments was 5 mM HEPES and 5 mM SDS. All buffers were made with Milli-Q deionized water and were filtered using a 0.2-μm filter. Proteins were used as received from Sigma and dissolved in 2.5 mM borax, pH 9.4. Derivatizing reagents, 5-furoylquinoline-3-carboxaldehyde (FQ) and potassium cyanide, were purchased from Molecular Probes (Eugene, OR). KCN was dissolved in 2.5 mM borax buffer at a concentration of 25 mM. A stock solution of 100 mM FQ was prepared in methanol, 10-μL aliquots were then placed into 500-μL microcentrifuge tubes, and the solvent was removed under vacuum using a Speed Vac (Savant Instruments Inc., Farmingdale, NY). The dried FQ aliquots were stored at −20 °C. These precautions were necessary since FQ degrades slowly in solution, even if the solutions are stored at −20 °C.

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Capillary Zone Electrophoresis. Derivatization was typically performed by adding 9 μL of protein solution and 1 μL of 25 mM KCN to a vial containing 100 nmol of previously dried FQ. Reaction times between 10 s and 1 h were used. Reaction vials were protected from light as much as possible.

Separations were performed in bare fused-silica capillaries at electric fields ranging from 400 to 700 V/cm. To reduce the background signal, the polyimide coating was removed from the last few millimeters of the capillary using a gentle flame. Samples were introduced electrokinetically.

Denaturing Gel Electrophoresis. Denaturing gel electrophoresis was performed with 10 μL of a solution that contained ovalbumin, BSA, conalbumin, myoglobin, and carbonic anhydrase, all at a concentration of 10^{-7} M. The solution was prepared in a 2.5 mM borax, pH ~9.4, containing 50 mM SDS and 1% 2-mercaptoethanol. The protein solution was denatured by heating at 95 °C for 5 min. This denatured protein solution was derivatized by mixing 7 μL of the solution with 100 nmol of dry FQ and 3 μL of a 5 mM KCN solution. The mixture was incubated for 10 min at room temperature. The solution was then diluted to 100 μL with deionized water. The same procedure was used for the blank except 2.5 mM borax was substituted for the protein solution.

The mixture was separated in a 7.5% dextran (MW = 2×10^6) solution that was prepared in a buffer made from 5 mM HEPES (pH 8.0) and 5 mM SDS. The capillary was coated with 3% polyacrylamide on a vinylmagnesium bromide sublayer according to the method of Cobb et al.[30] The capillary was equilibrated with the HEPES/SDS running buffer by applying a 1000 V/cm electric field for 10 min. The sample was injected for 5 s at 1000 V/cm; separation was also at 1000 V/cm. The dextran was replaced between each run.

Safety Precautions. Potassium cyanide is highly poisonous and reacts readily with acids to form lethal HCN gas. Stock solutions should be made in a basic buffer; neutralization of waste containing KCN should be made by addition of 1% NaOH (50 μL/g of cyanide) solution followed by slow addition of bleach (70 μL/g of cyanide).

RESULTS AND DISCUSSION

The most important parameter for efficient separations of labeled proteins is the concentration of SDS used in the separation buffer. More specifically, three regimes are present: free solution, submicellar, and micellar. These regimes are unique in terms of efficiency, mobility and resolution.

Efficiency and Mobility. (a) Free Solution. Ovalbumin is a useful model protein because it consists of several glycoforms with similar mobility; these glycoforms are resolved by free solution electrophoresis. Curve A in Figure 1 represents the free solution electropherogram of ovalbumin labeled with FQ using a 15-s reaction time and is compared to a UV absorbance electropherogram of unlabeled ovalbumin (curve B). The profiles are similar; the partially resolved peaks are glycoforms of ovalbumin.[31,32] The similarity in profile between electropherograms A and B demonstrates that broadening due to multiple labeling is not significant for the short incubation time. Presumably, only a few highly accessible sites are labeled during the first few seconds of reaction.

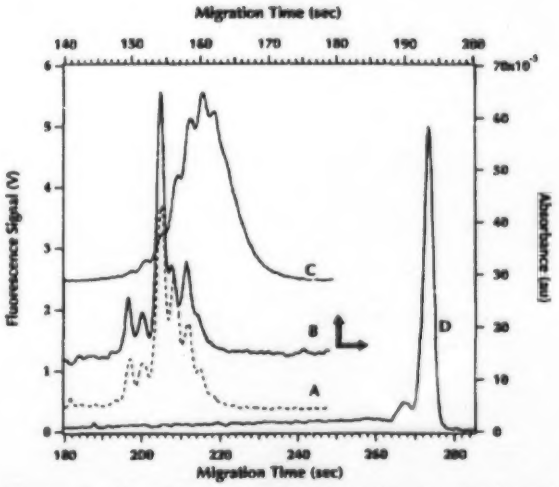

Figure 1. Comparison of ovalbumin analyzed under various conditions: running buffer (A–C) 25 mM tricine, pH 8.0; (D) 25 mM tricine + 5 mM SDS. (A) FQ-labeled ovalbumin, 15-s reaction, LIF detection, t_m = 205.2 s; (B) unlabeled ovalbumin, UV absorbance detection, t_m = 150.8 s; (C) FQ-labeled ovalbumin, 10-min reaction, LIF detection, t_m = 215.5 s, (D) FQ-labeled ovalbumin, 1-min reaction, LIF detection, t_m = 272.6 s. Note that electropherogram B is plotted vs top *x*-axis and right *y*-axis.

When the reaction time is increased to 10 min, broadening associated with multiple labeling increases and the glycoforms are no longer discernible (curve C). Presumably, a complex mixture of labeled products is produced, which generates a broad envelope under electrophoretic analysis. In this buffer system, which does not contain SDS, an increase in incubation time causes a loss of separation efficiency.

(b) Submicellar Separation. The high-efficiency separation is recovered by the addition of 5 mM SDS to the buffer (curve D in Figure 1). All information on glycosylation status is eliminated, and the complex and broad envelope of multiply labeled product peaks collapses to a single, narrow peak. The addition of SDS to the running buffer generated a similar improvement in efficiency for all proteins studied.

The interactions between SDS and proteins are complicated. SDS complexes with denatured proteins in polyacrylamide gel electrophoresis (SDS–PAGE) via interaction between the protein's hydrophobic regions and the long alkane chain of SDS. In addition, the anionic sulfate group of SDS interacts with positively charged side chains of unlabeled lysine residues.[33–36] Oakes demonstrated that, in the case of BSA, the binding of the first 20 SDS molecules occurs mainly through the anionic head group of SDS. The protein is not denatured at this concentration of SDS.[34] The conversion of lysine to a covalent FQ-lysine moiety or an ionic SDS-lysine moiety results in approximately the same increase in mass, 260 or 283 amu, respectively. In both cases, lysine is converted from positively charged to neutral. Therefore, the presence of SDS masks the mobility differences of the 1 048 575

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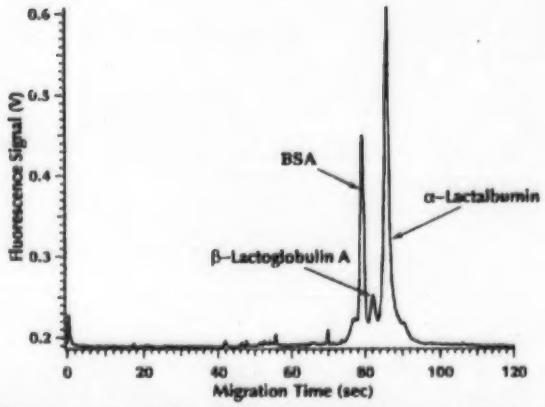

Figure 2. Separation of FQ-labeled bovine serum albumin, β-lactoglobulin, and α-lactalbumin labeled at a concentration of 5, 10, and 15 nM, respectively: 60-s reaction time, 5 s × 2 kV injection, 20 cm × 50 μm (i.d.) capillary, E = 700 V/cm, running buffer 2.5 mM borax + 5 mM SDS.

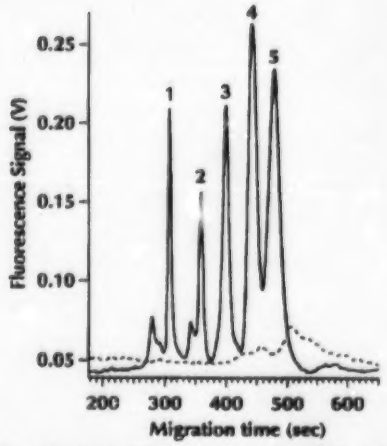

Figure 3. dCGE-LIF analysis of (1) myoglobin, (2) carbonic anhydrase, (3) ovalbumin, (4) BSA, and (5) conalbumin labeled at a concentration of 10^{-7} M with FQ. Solid trace, sample; dashed trace, blank. CE was performed on a 25 cm × 50 μm capillary coated with 3% polyacrylamide and filled with 7.5% dextran (MW = 2 × 10^6); E = 1 kV/cm.

possible products of the FQ ovalbumin labeling reaction. On the other hand, the interaction of SDS with the glycoforms remains obscure.

The SDS concentration, at 5 mM, is slightly below the cmc of 6.4 mM. The enhanced separation is not due to partitioning of the protein into and out of micelles, since there are no micelles present. Instead, the complexation of the SDS with the protein enhances efficiency.

(c) Micellar Separation. There is a gradual loss of separation efficiency as the surfactant increases above the cmc. The theoretical plate count for the analysis of labeled ovalbumin with a 5 mM SDS concentration, 140 000 plates, is 4 times larger than the plate count for the analysis with 20 mM SDS concentration.

Complexation between the protein and surfactant molecules generates high-quality separation at submicellar concentrations. In contrast, the much larger micelle will interact with the cationic lysine groups on the protein, creating a complex that is much larger than the FQ–lysine derivative.[33] Micellar separations are not efficient in masking mobility differences due to multiple labeling.

Separation of Proteins. (a) High-Speed, Submicellar Separation. The use of submicellar concentrations of SDS masks the differences in the electrophoretic mobility of multiply labeled proteins and different glycoforms of a protein, generating a high-efficiency electropherogram of a multiply labeled protein. More importantly, the addition of SDS does not degrade the separation of mixtures of proteins. Figure 2 shows the electropherogram of a mixture of FQ-labeled BSA, β-lactoglobulin, and α-lactalbumin labeled at 5, 10, and 15 nM, respectively. The resolution obtained is similar to that obtained by Saz et al. for the analysis of the native proteins.[12]

Our analysis took a total of 2.5 min; 1 min for derivatization and 1.5 min for separation and detection. The speed of the labeling process is important because a common criticism of precolumn labeling is the need for lengthy reactions. We have used 10-s reactions for concentrations of ovalbumin greater than 10^{-8} M. Reactions lasting less than 10 s should be possible but are difficult to perform manually. A capillary length of 20 cm and an electric field of 700 V/cm were used to obtain the electropherogram. With such short analysis times, the methods developed here should be useful for analyzing large number of samples for screening purposes or monitoring rapid changes in the levels of proteins.

(b) Denaturing Capillary Gel Separation. Free solution electrophoresis of proteins is a very simple technique. However, it is difficult to estimate the molecular weight of a protein based on its free solution mobility. Denaturing gel electrophoresis of unlabeled proteins produces a separation where the logarithm of molecular weight is proportional to the logarithm of migration time. We wish to demonstrate similar behavior for the fluorescently labeled proteins.

In denaturing capillary gel electrophoresis (dCGE), proteins are extensively denatured and treated with high concentration of SDS. The surfactant forms a complex with the protein, creating molecules with roughly identical size-to-charge ratio. The proteins are labeled, diluted 10-fold, and then separated in a dextran-filled capillary containing 5 mM SDS. Dextran acts as a sieving medium that separates the proteins based on size. Figure 3 shows the dCGE separation of (in order of elution) myoglobin, carbonic anhydrase, ovalbumin, BSA, and conalbumin labeled with FQ. A plot of log(migration time) vs log(molecular weight) was linear. The slope was 0.282 and the correlation coefficient (R^2) was 0.990.

Optimization of Reaction Conditions. The reaction time, the derivatizing reagent concentration, and the presence of organic solvents are critical factors in obtaining optimal sensitivity and efficiency. The presence of organic solvents is common because derivatizing reagents are often neither highly soluble nor stable in aqueous solutions. According to the manufacturer's protocol, stock solutions of FQ should be prepared in methanol and aliquots added to an aqueous solution of protein for derivatization. However, the presence of methanol in the derivatization step results in an additional, small, broad and partially resolved peak prior to the main peak (Figure 4). With the elimination of methanol from the reaction mixture, the broad peak is almost completely eliminated and efficiency increases from 30 000 (electropherogram A) to 190 000 plates (electropherogram C). Bull

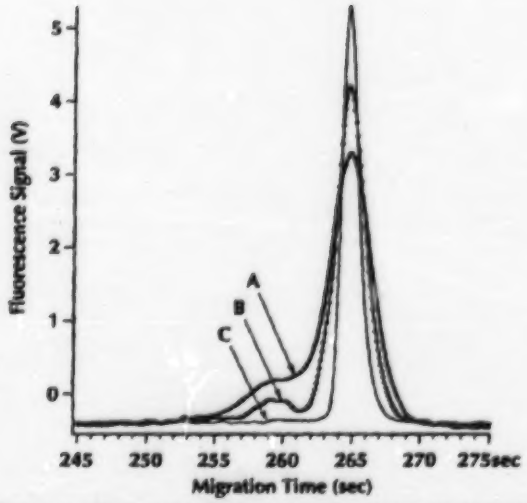

Figure 4. Effect of the presence of methanol on peak efficiency. Reaction conditions: (A, B) 10^{-8} M ovalbumin, 1-min reaction, 1:20 dilution in running buffer, 5 s × 3 kV injection; (C) 10^{-9} M ovalbumin, 26-min reaction, 5 s × 5 kV injection. Percent MeOH in reaction: (A) 75, (B) 2.5, and (C) 0%. Peaks offset slightly for clarity; actual migration times were 264.6 s in (A), 263.7 s in (B), and 259.5 s in (C). CE conditions for all runs: running buffer, 2.5 mM borax + 5 mM SDS; E = 400 V/cm

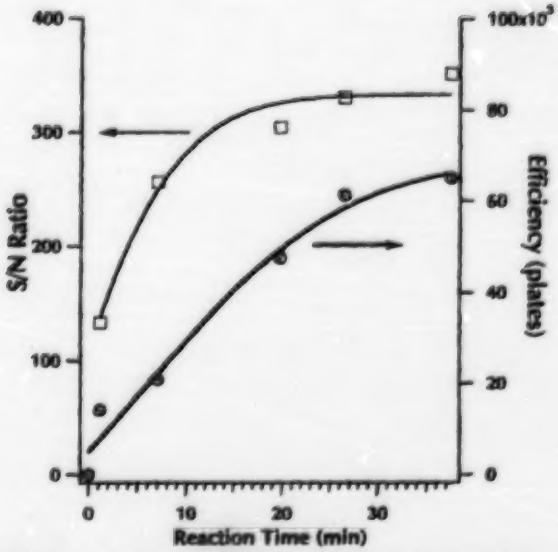

Figure 5. Dependence of signal-to-noise ratio (circles) and peak efficiency (squares) on reaction time. Reaction conditions: 14.4 μL × 10^{-9} M ovalbumin + 1.8 μL × 25 mM KCN + 80 nmol FQ, 5 s × 5 kV injection, 48 amol injected. CE conditions, same as Figure 4

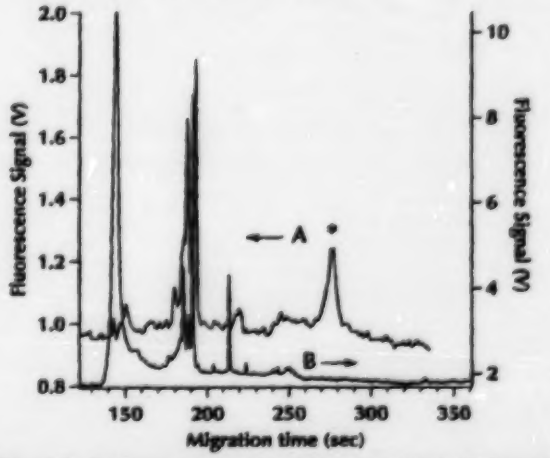

Figure 6. Picomolar analysis of conalbumin and blank. (A) Sample reaction conditions: 2 μL × 10^{-10} M conalbumin + 1 μL × 10 mM KCN + 2 μL × 10 mM FQ, 17.5-min reaction, 5 s × 5 kV injection (177 mol injected), S/N = 51. FQ-conalbumin peak indicated with asterisk, other peaks due to secondary reaction products. (B) Blank reaction conditions: 2 μL × 2.5 mM borax + 1 μL × 10 mM KCN + 2 μL × 10 mM FQ, 17-min reaction, 5 s × 10 kV injection. CE conditions, same as Figure 4

and Breese studied the denaturing effects of alcohols on various proteins.[35] They hypothesized that denaturation is accompanied by dehydration. In the case of methanol, they found that the denaturation reaction was first order in protein concentration and 18th order in methanol concentration. This fact may explain why as little as 2.5% (v/v) methanol is sufficient to cause denaturation (Figure 4, electropherogram B).

Reaction time plays a critical role in optimizing sensitivity and efficiency (Figure 5). The signal-to-noise ratio and the peak efficiency reach a plateau after ~30 min. This increase in efficiency with reaction time is only observed with buffers that contain SDS near the cmc.

Detection Limit. It is important to distinguish between assay and instrumental detection limit. The assay detection limit refers to the total process of labeling and detection and represents the minimum amount of *unlabeled* protein required for analysis. The instrumentation detection limit refers to the detection of labeled protein and ignores difficulties in labeling dilute solutions of proteins.

Fluorogenic derivatizing reagents tend to produce low background signals. Figure 6, curve B, presents the submicellar electropherogram of a reagent blank. The blank generated several peaks with a migration time of ~180 s. The background signal appears to be due to trace impurities present in the derivatizing reagent. The shape of the background signal changes from lot-to-lot of reagent, presumably due to slight differences in the composition of these impurities. Fortunately, none of the proteins that we have analyzed comigrate with these background peaks.

FQ may be used to assay picomolar concentrations of protein. An electropherogram of conalbumin *derivatized* at a concentration of 10^{-10} M is shown as curve A in Figure 6. The assay detection limit (3σ) for unlabeled conalbumin is 1 × 10^{-11} M (Table 1). These are among the lowest reported mass and concentration detection limits for a native protein using any detection scheme for CE. These detection limits reflect the instrumental detection limits along with the reaction efficiency, impurity production, adsorption losses to the vessel walls, etc.

The assay detection limits can be no better than the instrumental detection limits. To estimate the instrumental detection limit, 1.0 μM conalbumin was labeled with FQ for 55 min and then diluted to 83 pM with running buffer. The sheath flow cuvette generated mass detection limits of 560 molecules (930 ymol) of FQ-labeled conalbumin injected onto the capillary in an

Table 1. Comparison of Limits of Detection (LODs)

protein	MW (×10³)	Lys/Trp/Tyr/Phe (residues)	concn LODs (×10⁻⁹ M) multiple-label[a] LIF	TOA[b]	native[c] LIF
trypsin inhibitor	7.5	10/2/4/9	1	27000	–[d]
α-lactalbumin	14.2	12/4/4/4	0.5	3700	–
myoglobin	17.2	19/2/3/6	0.5	63	–
β-lactoglobin A	19.9	15/2/4/4	1	4000	–
carbonic anhydrase	31	20/6/10/11	0.5	100	–
ovalbumin	45	20/3/10/20	0.1	–	–
bovine serum albumin	69.2	60/3/21/30	0.05	10	0.3
conalbumin	77.8	59/11/21/27	0.01	–	0.15

[a] This work (detection limits are at a S/N 3). [b] See ref 12 (detection limits were converted to S/N 3). [c] See ref 9 (detection limits were converted to S/N 3). [d] –, not given.

injection volume of 2.9 nL and assuming 100% labeling efficiency. The instrumental detection limit is 3.3×10^{-13} M. The instrumental detection limit of FQ-labeled protein is 30 times lower than the assay detection limit of unlabeled protein.

Linear Dynamic Range for Bovine Serum Albumin. A log–log calibration curve for BSA was generated for labeling reactions performed over a concentration range from 5×10^{-10} to 1×10^{-5} M using a 30-min reaction time. The curve was linear over 5.5 orders of magnitude, with a slope of 1.11 ± 0.06 and a correlation coefficient of 0.9994. At concentrations below 5×10^{-10} M, insufficient product was formed to permit detection. This detection limit for BSA is 5-fold poorer than that observed for conalbumin. The two proteins have nearly the same number of lysine residues; therefore, the difference in detection limit must result from differences in reaction efficiencies of the two molecules. Apparently, BSA does not have a large number of lysine residues that are readily accessible to the derivatizing reagent.

At concentrations equal to or higher than 10^{-4} M, the calibration curve deviated from linearity. At a BSA concentration of 10^{-4} M, the FQ/lysine ratio is only 1.7 and the CN^-/lysine ratio is only 0.42. Therefore, the concentration of both derivatizing reagents decreases significantly during the reaction, causing a negative deviation from linearity. The solubility of FQ limits the use of higher concentrations in aqueous solutions.

Comparison of LIF Detection and UV Absorbance Detection. The electropherograms shown in Figure 7 compare the signal-to-noise ratio of LIF and UV absorbance detection. The protein was derivatized with FQ in the case of LIF detection. Relative signal-to-noise ratios were calculated for the three detection methods after correcting for the different amounts injected (Table 2). Sheath flow LIF detection provides the highest signal-to-noise ratio and is assigned a relative value of 1. The signal-to-noise ratio of on-column LIF detection is 80 times poorer than that of sheath flow LIF detection while the signal-to-noise ratio of UV absorbance detection is 30 000 times poorer than the sheath flow LIF detector.

Comparison of High-Sensitivity Protein Detection Schemes for CE. Table 1 contains the concentration detection limits obtained for various proteins using sheath flow LIF detection as well as literature values for TOA detection[12] and native LIF detection,[9] all data converted to S/N = 3. In the TOA detection study, the samples were dissolved in water. Therefore, the TOA detection limits may have been enhanced due to on-column

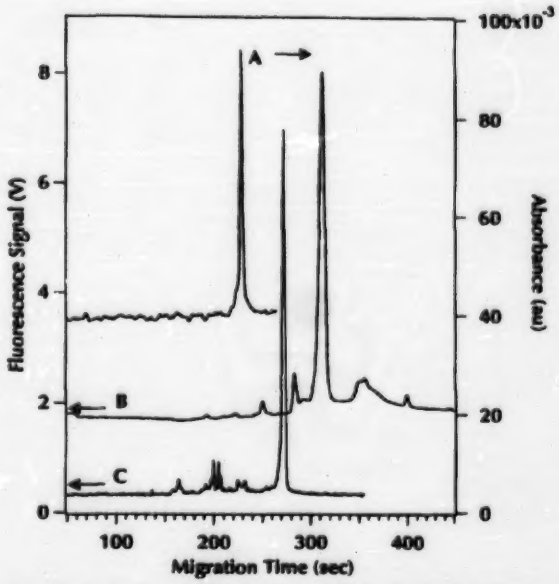

Figure 7. CE analysis of ovalbumin. (A) UV absorbance detection [ovalbumin] = 10^{-4} M, 48.1 cm × 50 μm (i.d.) capillary, 5 s × 1 kV injection; (B) on-column CE-LIF detection, [ovalbumin] = 10^{-6} M, 57 cm × 75 μm (i.d.) capillary, 5 s × 1.5 kV injection; (C) sheath flow CE-LIF detection, [ovalbumin] = 10^{-6} M, 50 cm × 50 μm (i.d.) capillary, 5 s × 5 kV injection. Arrows indicate appropriate y axis. CE conditions, same as Figure 4

Table 2. Comparison of UV and LIF Detection

detection method	ovalbumin concn (M)	amt injected (amol)	relative S/N	efficiency (plates)
on-column UV abs	10^{-4}	110000	0.000033	28 000
on-column LIF	10^{-6}	670	0.012	20 000
sheath flow LIF	10^{-9}	41	1.0	52 000

stacking. The authors of the native fluorescence study lowered their concentration detection limits to 3 pM for conalbumin and 11 pM for BSA by using stacking; however, they also reported results for analysis without stacking that are listed in Table 1.[9] The detection limits reported here are 10–10 000 times lower than other detection schemes for the CE analysis of proteins, at least in the absence of stacking. It should be mentioned that, for precolumn labeling, stacking only improves detection limits if the analyte and background species do not coelute since stacking also concentrates the background species.

The proteins' molecular weight and the number of lysine (Lys), tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues[37,38] are essential in understanding the differences in sensitivity between the techniques. The absorption of light, and its conversion to heat, is the basis of TOA detection. High sensitivity is only obtained for proteins that have a large number of the three highly absorbing residues, namely, Phe, Tyr, and Trp. The molar absorptivities for Phe, Tyr, and Trp are 197, 450, and 3500 M^{-1}

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cm^{-1}, respectively, for the free amino acids at 257 nm, which is the wavelength used in the TOA study.[39]

Tryptophan fluorescence is the basis of native LIF detection, although in some cases tyrosine fluorescence is important. Tyrosine only fluoresces in the absence of tryptophan, and even then only with low quantum yield.[40] Small species, such as O_2, I^-, and acrylamide quench tryptophan's fluorescence. Also, tryptophan's quantum yield decreases by two-thirds when the residue is in a hydrophobic environment as compared to the quantum yield of tryptophan residues in a hydrophilic environment.[41] In proteins, approximately a third of the tryptophan residues are located in the hydrophobic regions.[42] Also, tryptophan's low abundance, at only 1.3/100 residues,[43] indicates that many small proteins would escape detection by native fluorescence. The detection limits based on native fluorescence shown in Table 2 correspond to two proteins that have a large number of tryptophan residues and are thus particularly suited to this detection scheme.

Finally, in the case of precolumn derivatization, detection is based on fluorescence of FQ-labeled amino groups, either the ϵ-amino group of lysine or the α-amino group of the N-terminal amino acid. Lysine, whose abundance is 5.7/100 residues, is one of the more abundant amino acids.[43] Highest sensitivity is obtained for proteins that have a large number of lysine residues such as conalbumin, which has 59 lysine residues.

In addition to sensitivity, the universality of the detection scheme and the cost of the detector are important. The universality and limitations of TOA, native LIF, and multiple-label LIF are largely based on the ratios of the four residues. The average ratio of Lys/Trp/Tyr/Phe residues in eukaryotic proteins is 4.4/1/2.5/3.[44] Therefore, of the three detection schemes, multiple-label LIF detection should be the most universal. Practically, the deep-UV argon ion lasers used in the native fluorescence and thermooptical studies costs many tens of thousands of dollars, require water cooling, require three-phase 220-V electrical power, and have a relative short lifetime; the argon ion laser used in this study costs less than U.S. $10,000, is air-cooled, operates from 110 V, and has a long lifetime.

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Douglas B. Craig*
Robert M. Polakowski
Edgar Arriaga
Jerome C. Y. Wong
Hossein Ahmadzadeh
Costas Stathakis
Norman J. Dovichi*

Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada

Sodium dodecyl sulfate-capillary electrophoresis of proteins in a sieving matrix utilizing two-spectral channel laser-induced fluorescence detection

We report a method for protein labeling, separation by capillary electrophoresis in a polymer sieving matrix, and detection by laser-induced fluorescence. Different dyes are used to label standard and sample proteins. A two-spectral channel detector resolves fluorescence from the sample and standards. Comparison of the migration time of the sample and standards permits the precise determination of molecular weight, irrespective of variations in run-to-run migration times.

1 Introduction

Sieving polymer-based separations of proteins under denaturing conditions exploit the binding of SDS to proteins at a ratio of 1.4 g SDS to 1 g protein [1, 2]. The binding of SDS swamps the native charge of proteins, making them highly negative, which results in a near-constant charge-to-mass ratio and which generates poor resolution when analyzed in free-zone electrophoresis. In contrast, SDS-proteins are separated based on their size alone by electrophoresis through a sieving matrix. SDS-PAGE is widely used for protein analysis, utilizing a crosslinked polyacrylamide slab gel. Several samples can be run on a single gel in different lanes, and the molecular weight of the sample is estimated based on a comparison with the separation of a series of size markers. After separation, the proteins are stained and the amount of protein can be semi-quantitatively determined by densitometry. The simple technique provides an estimate of the molecular weight of the protein with ~10% accuracy, where accuracy refers to agreement with the true value. The technique also requires relatively large amounts of proteins. This method has been modified for use in capillary electrophoresis using a polymer sieving matrix in place of the polyacrylamide gel [3–6]. CE provides online detection, avoiding the time-consuming steps of staining and destaining. Additionally, CE allows separation at a high electric field, resulting in fast separations. Unfortunately, only one sample can be run at a time per capillary; one capillary is the equivalent of a single lane in an SDS-PAGE gel.

Reproducible estimates of the molecular weight are difficult in CE. Conventionally, the sample and standards are analyzed sequentially on the same column. The migration times of the sample and the standards are compared to estimate the molecular weight of the sample. Variations in migration time arise from changes in the capillary temperature, running buffer composition, or electroosmotic flow in the capillary; these variations degrade the precision of the molecular weight estimate by CE. Internal standardization techniques have been described that are based on the relative migration time between analyte and standard compounds [7]. These techniques have been applied to one-color optical detection so the standards must not comigrate with analyte. In this paper, we report a two-color protein analysis system, wherein samples and standards are analyzed simultaneously on the same capillary. Samples are labeled with one fluorescent dye and standards are labeled with a second dye; by use of the two dyes and a two-color detector, sample and standard can be independently detected in separate channels, minimizing difficulties associated with comigration of sample and standard. The molecular weight of bovine serum albumin is estimated with 1% reproducibility, which is a tenfold improvement over a conventional sequential analysis.

2 Materials and methods

2.1 Instrumentation

Details of the instrument have been published previously [8–11]. The injection end of a coated capillary was immersed into the sample or running buffer along with a platinum wire connected to a high voltage power supply (CZE1000R, Spellman, Plainview, NY, USA). The polyimide coating at the detection end of the capillary was removed by a gentle flame, while continuously flushing buffer through the capillary, and placed inside a 250 × 250 μm inner bore sheath flow cuvette. Analyte exiting the capillary was ensheathed by a flowing buffer stream and was forced to pass through the center of the cuvette. Migrating species were excited by a 488 nm beam from an Ar^+ laser (Coherent, Palo Alto, CA, USA) focused 20 μm below the capillary end by a 6.3 × 0.20 NA microscope objective (Melles Griot, Nepean, ON, Canada). The laser power was 10 mW. Fluorescence was collected at right angles by a 60 × 0.70 NA microscope objective (Model 60x-LWD, Universe Kogaku, Japan) and passed through a slit and onto a 580 nm dichroic filter (Omega Optical, Brattleboro, VT, USA). Emission at wavelengths shorter than 580 nm was transmitted through this filter, then filtered by a 513DF25 bandpass filter (Omega Optical), and detected with an R1477 photomultiplier tube

Correspondence: Professor Norman J. Dovichi, Department of Chemistry, University of Alberta, Edmonton, Alberta T6G2G2, Canada (Tel: +403-492-2845, Fax: +403-492-8231, E-mail: norm.dovichi@ualberta.ca)

Abbreviations: **FQ**, 3-(2-furoyl)quinoline-2-carboxaldehyde; **FX**, 6-(fluorescein-5-carboxamido) hexanoic acid succimidyl ester; **PMT**, photomultiplier tube, **THF**, tetrahydrofuran

Keywords: Protein separation / Sieving matrix / Sodium dodecyl sulfate / Capillary electrophoresis

* Current address: Department of Chemistry, University of Winnipeg, Winnipeg, Manitoba, R3B 2E9 Canada

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(PMT; Hamamatsu, Middlesex, NJ, USA). Emission at wavelengths longer than 580 nm was reflected from the dichroic, then filtered with a 630DF30 bandpass filter, and detected with a second PMT. The analog PMT signal was digitized at 10 Hz by a Macintosh IIsi *via* an NB-MIO-16XH-18 I/O board (National Instruments, Austin, TX, USA). The board also controls the CE power supply.

2.2 Materials

Conalbumin (78 000 Da), bovine serum albumin (BSA, 69 000 Da), ovalbumin (45 000 Da), trypsinogen (7500 Da), and dextran were obtained from Sigma (St. Louis, MO, USA). 3-(2-Furoyl)quinoline-2-carboxaldehyde (FQ) and 6-(fluorescein-5-carboxamido)hexanoic acid succimidyl ester (FX) were supplied by Molecular Probes (Eugene, OR, USA). Tetrahydrofuran (THF) and vinylmagnesiumbromide were purchased from Aldrich (Milwaukee, WI, USA) and NaCN, SDS, and disodium tetraborate from BDH (Toronto, ON, Canada). HEPES was from Fisher (Ottawa, ON, Canada) and $NaHCO_3$ from Baker (Philipsburg, NJ, USA). TEMED and ammonium persulfate were from Bio-Rad (Hercules, CA, USA) and acrylamide from GibcoBRL (Gaithersburg, MD, USA). Polyethylene oxide was supplied by Polysciences (Warrington, PA, USA).

2.3 FX-labeling of standards

A 10 µL solution of 1 mM trypsinogen, ovalbumin, or conalbumin was made in 100 mM $NaHCO_3$ and mixed with 1 µL of 10 mM FX in DMSO. The solution was incubated at room temperature for 30 min. Reaction mixtures were pooled and the unincorporated FX and side products were removed by passing the sample through a Sephadex G-25 F column equilibrated with 5 mM borate, 5 mM SDS, 1.5 mM NaCN and 1% v/v 2-mercaptoethanol, pH 9.3. Dilutions were made in the same buffer. The sample was incubated at 100°C for 3 min prior to injection.

2.4 FQ-labeling

Protein solutions were prepared at 10^{-4} or 10^{-7} M in 5 mM borate, 5 mM SDS, 1.5 mM NaCN and 1% 2-mercaptoethanol, pH 9.3, and incubated at 100°C for 3 min. A sample volume of 10 µL was added to 100 nmol dry FQ and incubated at room temperature for 30 min.

2.5 Capillaries

Capillaries were coated using the method of Cobb *et al.* [11]. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ; 50 µm ID, 145 µm OD, 5 m long) were preconditioned by flushing by nitrogen pressure (20 psi) with 1 M NaOH for 3 h, followed by distilled water for 1 h and methanol for 1 h. Capillaries were dried overnight at 120°C, flushing with nitrogen at 5 psi. Temperature was reduced to 65°C and the capillaries were flushed with thionyl chloride under 20 psi pressure for 30 min. The ends of the capillary were capped and the unit was heated for 8 h at 65°C. The capillary was then uncapped, filled with anhydrous tetrahydrofuran (THF) and flushed 30 min at 20 psi pressure with 0.34 M vinylmagnesiumbromide in THF. The capillary end was capped and the capillary heated for 8 h at 65°C. The capillary was uncapped and flushed with anhydrous THF for 30 min at 20 psi followed by distilled water for 30 min. A solution prepared with 2 µL TEMED, 20 µL 10% ammonium persulfate and 5 mL 3% acrylamide (no cross-linker) was flushed through the capillary at 20 psi for 10 min. The capillary was cut to the desired length and flushed with distilled water followed by running buffer prior to use.

2.6 Capillary electrophoresis

The capillary (28 cm long) was manually flushed with 5 mM HEPES, 5 mM SDS, pH 8.0, containing 7.5% w/v dextran (2 MDa average molecular weight). The capillary was equilibrated by CE at 500 Vcm^{-1} prior to sample injection. The polymer was replaced and the capillary equilibrated prior to each run. A 10 µL solution of 10^{-7} M BSA was labeled with FQ and spiked with 1 µL FX-labeled protein standards to a final concentration of 1.8×10^{-4} M. The sample was electrokinetically injected for 5 s at 500 Vcm^{-1}. CE was performed at 500 Vcm^{-1} using 5 mM HEPES containing 5 mM SDS, pH 8.0, as running buffer. Sheath buffer was 5 mM HEPES containing 5 mM SDS, pH 8.0.

3 Results and discussion

Fluorescence labeling of proteins provides two advantages over UV absorbance detection. First, picomolar concentrations of proteins can be labeled with fluorogenic reagents and detected by laser-induced fluorescence, which provides over a millionfold higher sensitivity than UV absorbance detection [13]. Second, as we show below, different labels can be used for sample and standard; the spectral signatures of the two dyes can be used to determine the migration time of the sample and standards. A molecular weight size curve can be generated based on the migration time of the standards. The molecular weight of the sample is then estimated based on its migration time. Because the sample and standards are run simultaneously, drift in migration time between runs does not degrade the precision of the molecular weight estimate. FQ is a useful reagent to label protein samples [13, 14]. The fluorogenic reagent is nonfluorescent until it reacts with a primary amine and the unreacted reagent generates a very low background signal. FQ-labeled proteins may be excited by an argon ion laser beam at 488 nm. The products have an emission maximum at 590 nm; we detect fluorescence at 630 nm to minimize interference from the fluorescein emission.

Figure 1a shows an electropherogram of 10^{-4} M FQ-labeled trypsinogen, ovalbumin, and conalbumin. Fluorescence was excited at 488 nm and the emission was monitored simultaneously at both 630 and 513 nm. FQ-labeled proteins emit strongly at 630 nm and generate little signal in the 513 nm band. Log(migration time) increased linearly with log(molecular weight), $r = 0.989$. We use fluorescein to label our standards; the dye

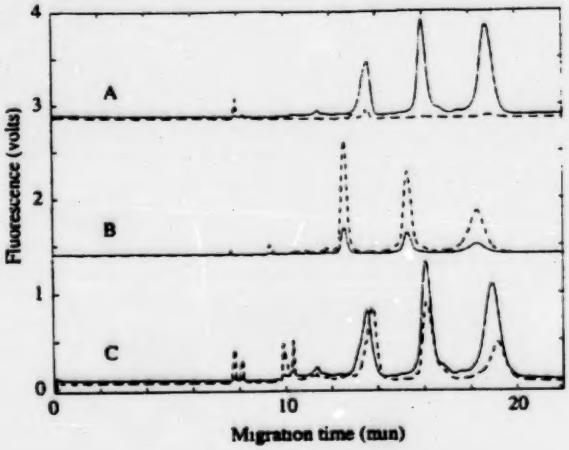

Figure 1. Separation of fluorescently labeled trypsinogen, ovalbumin and conalbumin. Separation was in a 28 cm long, 50 μm ID, 145 μm OD coated capillary filled with 5 mM HEPES/5 mM SDS, pH 8.0, containing 7.5% w/v dextran (average molecular mass 2MDa) at an electric field of 500 Vcm^{-1}. Running and sheath buffer was 5 mM HEPES/5 mM SDS, pH 8.0. Sample buffer was 5 mM borate/5 mM SDS/1.5 mM NaCN/1% 2-mercaptoethanol, pH 9.3. Detection was by laser-induced fluorescence. Excitation was at 488 nm and emission was measured at 630 and 513 nm. All data were treated with a median filter over 2.8 s to remove the effect of bubbles on the signal. The three traces were offset for clarity. (A) 10^{-6} M proteins were labeled 30 min with FQ and injected. Trypsinogen migrates the fastest, followed by ovalbumin and conalbumin. The solid line shows the signal at 630 nm and the broken line at 513 nm. (B) 10^{-3} M proteins were labeled with FX, the unincorporated label removed and the sample diluted to 5×10^{-8} M prior to injection. Migration order was identical to (A). The solid lines gives the signal at 630 nm and the broken line at 513 nm. (C) 10^{-6} M proteins were labeled with FQ and the sample was spiked with FX-labeled protein to final concentration of 4.5×10^{-8} M. Migration order was identical to (A). The solid line gives the signal at 630 nm and the broken line at 513 nm.

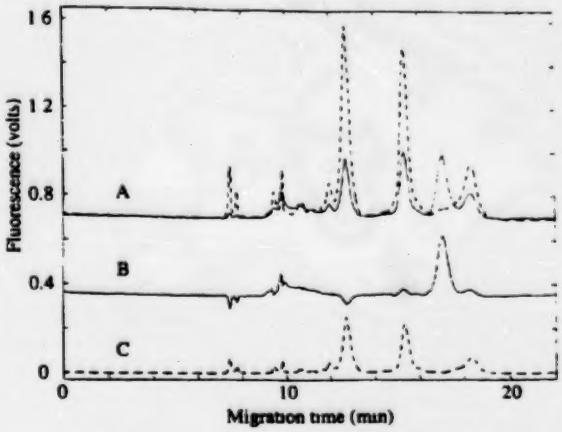

Figure 2. Determination of the molecular weight of BSA. 10^{-7} M BSA was labeled with FQ and the sample was spiked with FX-labeled trypsinogen, ovalbumin and conalbumin to a final concentration of 1.8×10^{-8} M. Capillary electrophoresis was as in Fig. 2. All data were treated with a median filter over 2.8 s to remove the effect of bubbles on the signal. (A) The signals at 630 nm (solid line) and 513 nm (broken line) are shown. (B) The signal generated by FQ-labeled BSA has been isolated from the raw data of the top panel by use of the matrix-inversion algorithm described in Section 3. (C) The signal generated by FX-labeled standard proteins has been isolated from the raw data of the top panel by use of the matrix-inversion algorithm described in Section 3.

is efficiently excited by the argon ion laser and its emission at 520 nm is easily spectrally resolved from the FQ emission of the labeled sample. The succinimidyl ester of the dye, particularly when used with an alkyl linker arm, is reasonably efficient at labeling proteins; the dye is denoted by the abbreviation FX. The fluorescent standards are prepared at a relatively high concentration, purified, and then diluted for use as size markers. Figure 1b shows an electropherogram resulting from the injection of trypsinogen, ovalbumin and conalbumin labeled with FX at 10^{-3} M and diluted to 5×10^{-8} M prior to injection. FX-protein conjugates produce strong fluorescence at 513 nm with some signal observed at 630 nm. Log(migration time) increased linearly with log(molecular weight), $r = 0.993$. Figure 1c shows an electropherogram resulting from injection of a sample containing both FX- and FQ- labeled trypsinogen, ovalbumin, and conalbumin. Proteins were labeled at 10^{-6} M with FQ and spiked with FX- labeled proteins to a final concentration of 4.5×10^{-8} M immediately prior to injection. The 513 nm channel primarily shows fluorescence from FX-labeled protein whereas the 630 nm channel shows fluorescence from FX- and FQ-labeled protein. FX- and FQ-labeled proteins have similar but not identical migration times, with FX-trypsinogen and FX-conalbumin having slightly longer migration times than their FQ-labeled counterparts.

Figure 2 shows an electropherogram generated by labeling a 10^{-7} M solution of FQ-labeled BSA and then spiking the sample with FX-labeled trypsinogen, ovalbumin, and conalbumin to a final concentration of 1.8×10^{-8} M. Five runs were performed in total. The top plot shows both the 513 nm and 630 nm traces. After background subtraction, the fluorescence signal in either spectral channel is generated by a linear combination of the intensity of the two dyes:

$$I_{513} = [FX] \times \alpha + [FQ] \times \beta$$

$$I_{630} = [FX] \times \gamma + [FQ] \times \delta \quad (1)$$

where I_{513} and I_{630} are the fluorescence intensity at these two wavelengths, and α, β, γ, and δ are constants that relate the concentration of dye to the fluorescence signal in each channel. This equation may be written in matrix form

$$\begin{bmatrix} I_{513} \\ I_{630} \end{bmatrix} = \begin{bmatrix} \alpha & \beta \\ \gamma & \delta \end{bmatrix} \times \begin{bmatrix} [FX] \\ [FQ] \end{bmatrix} \quad (2)$$

The matrix equation can be inverted; the result is used to convert the fluorescence intensity traces to the concentration of the dye-labeled proteins

$$\begin{bmatrix}[FX]\\[FQ]\end{bmatrix}=\begin{bmatrix}\alpha & \beta\\ \gamma & \delta\end{bmatrix}^{-1}\times\begin{bmatrix}I_{513}\\ I_{630}\end{bmatrix} \quad (3)$$

Trace (B) in Fig. 2 was obtained by calculating the concentration of FQ-labeled sample proteins while trace (C) was obtained by calculating the concentration of FX-labeled standards. The linear algebra of Eqs. (1) and (2) assumes that the fluorescence spectrum of each dye-labeled protein is the same for all proteins. This assumption does not appear to be correct for FX, where the earliest migrating proteins appear to generate a larger red signal than the larger, later migrating proteins. We believe that the larger proteins contain several fluorescent labels and that these labels are stacking and forming dimers. The resulting dimer fluorescence is red shifted with respect to the monomer emission. Because of the slight differences in the fluorescence spectrum of the FX-labeled proteins, the matrix inversion algorithm generates baseline disturbances in the FQ trace. Migration times for individual peaks varied by an average RSD of 3% for the five runs. Log(migration time) *vs.* log(molecular weight) plots of the standards for each run yielded an average r of 0.999. Calculated molecular weights of BSA, based on the migration times of the standards for the particular runs, ranged from 61.3 to 62.5 kDa, with an average of 62.0 kDa and an RSD of < 1%. The use of internal size standards provides precise estimates of the molecular weight of the sample protein.

If we had not used internal standards, the reproducibility in migration time would have been much poorer. To demonstrate the precision obtained by the use of separate sample and standard electropherograms to estimate the molecular weight of a sample protein, we averaged the migration time of the standards in the five runs, and we used these values to construct a size curve. The sample migration time from each run was compared with the size curve and was used to estimate the molecular weight of BSA. Calculated molecular weights ranged from 50.7 to 71.5 kDa with an average of 62.2 kDa and an RSD of 11%. The use of separate runs to estimate molecular weight leads to poor reproducibility. The accepted molecular weight of BSA is 66 kDa. SDS-PAGE typically estimates protein size with an accuracy of 10% [1, 2]. The use of internal standards in a capillary electrophoresis system gives a precise value for the size of BSA and an accuracy that is comparable to that produced by SDS-PAGE. Average theoretical plate counts were 7300 for trypsinogen, 11000 for ovalbumin, 7100 for BSA, and 5200 for conalbumin ($n = 5$). Separation efficiencies are considerably less when compared to the plate counts obtained with detection of unlabled proteins by UV absorbance, and may reflect multiple labeling effects or residual adsorption of the protein to the capillary wall.

The method we describe allowed detection and separation of proteins at concentrations less than 10^{-7} M. For comparison, UV detection on the commercial instrument failed to detect peaks at concentrations below 10^{-4} M. The method presented here is less sensitive than that described by Pinto *et al.* [13], who report the FQ labeling and detection of 10^{-10} M protein in the absence of SDS or a sieving matrix. To denature the proteins, we boiled the sample in the presence of SDS and 2-mercaptoethanol prior to injection. We found that sample heating in the presence of the FQ dye resulted in a high and irreproducible background fluorescence signal. Much more reproducible results were generated by labeling the protein after denaturation. The poorer sensitivity from post-denaturation labeling probably arises because the labeling reagents are both electrostatically repulsed and sterically hindered by the heavy coating of SDS on the protein surface. Finally, sensitivity also degrades for the broad peaks generated in this separation. These broad peaks arise, in part, from multiple labeling of the protein [15], where each of the free amines in the protein is available for labeling. Incomplete labeling leads to a huge number of possible reaction products. Pinto [13] demonstrated much higher efficiency separations by use of submicellar capillary electrophoresis. The narrow peaks have much higher amplitude for a given amount of protein than do the broad peaks we generated here.

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Analytical Chemistry
in press

Picomolar Analysis Of Proteins Using Electrophoretically Mediated Microanalysis And Capillary Electrophoresis With Laser Induced Fluorescence Detection

In Ho Lee[†], Devanand Pinto, Edgar A. Arriaga, Zheru Zhang, and Norman J. Dovichi[*].
Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada, T6G 2G2.

Abstract

We report a method for the analysis of picomolar concentration proteins by using electrophoretically mediated microanalysis (EMMA) to label proteins on-column with a fluorogenic reagent. Labeling is followed by capillary zone electrophoresis separation and post-column detection based on laser-induced fluorescence. The method provides a concentration detection limit (3 σ) of 3×10^{-13} M for conalbumin. The method provides separation efficiency of 400,000 theoretical plates. Protein extract from a human colon adenocarcinoma cell line generated a dozen major components and many minor components in a 12 minute separation; the protein extract from 2.5 cells was used for this analysis. When compared to UV absorbance detection, the EMMA method provides 7,000,000 fold improvement in detection limit and a 13 fold improvement in efficiency.

[†] Present Address, Department of Chemistry, Taejon University, Taejon, 300-716, Korea

* Author to whom correspondence should be addressed.

Capillary electrophoresis is a powerful separation tool for proteins. However, conventional ultraviolet absorbance detection produces poor concentration detection limits, which prohibits the use of capillary electrophoresis for trace-level analysis. Laser-induced fluorescence can provide highly sensitive detection of very low concentrations of native and derivatized proteins (1-12). Native fluorescence detection usually requires the use of expensive and temperamental lasers that operate in the ultraviolet portion of the spectrum. Derivatized proteins can be detected with relatively inexpensive air-cooled argon ion or helium-neon lasers; however, derivatization chemistry can suffer not only from interference due to excess derivatizing reagent but also from complex electropherograms generated by multiple reaction products due to incomplete labeling of all possible sites on the protein (13-14).

Fluorogenic reagents have proven useful for protein analysis (15). These reagents are not fluorescent until they react with a primary amine; they generate low background signals compared to traditional fluorescent derivatizing reagents. We have exploited 3-(2 furoyl)quinoline-2-carboxaldehyde (FQ) to generate highly fluorescent proteins that are separated efficiently when used with a submicellar buffer system, without noticeable band-broadening due to multiple labeling (11). We reported the successful labeling and analysis of a 10^{-11} M conalbumin by this method.

Unfortunately, most labeling chemistry is cumbersome and difficult to automate. In this paper, we report the automated reaction between protein and FQ in the capillary as a form of electrophoretically-mediated microanalysis (EMMA) (16-20). In EMMA, reagents are introduced in order of increasing mobility. Under the influence of an electric field, the reagents mix and react to form a product. The time during which the reagent zones overlap determines the reaction time, which can be controlled by stopping the electric field after the reagent zones mix. After the reaction is complete, the product is separated from the reagent using zone electrophoresis. By performing the reaction at 65 °C rather than room temperature, we obtain a tenfold improvement in detection limit for conalbumin and reduce the total analysis time from 35 to 5 minutes. Picomolar concentration and zeptomole amounts of protein are analyzed by EMMA-CE-LIF.

EXPERIMENTAL

Reagents

Sodium tetraborate (borax), sodium dodecyl sulfate (SDS), and sodium pentasulfonate (SPS) were from BDH (Toronto, ON, Canada). All buffers were made with Milli-Q deionized water and were passed through a 0.2-μm filter. All proteins were from Sigma (St. Louis, MO). The proteins were used as received. Protein solutions were prepared daily in 2.0 mM borax, 4 mM SDS, 5 mM KCN. The final concentration of proteins varied from 10^{-8} M to 10^{-12} M. All solutions were kept on ice. Derivatizing reagents, FQ and potassium cyanide, were from Molecular Probes (Eugene, OR). KCN was dissolved in water (100 mM).

FQ solutions were dried before storage. A stock solution of 100 mM FQ was prepared in methanol, 10 μL aliquots were placed into 500 μL microcentrifuge tubes, and the solvent was removed under vacuum using a Speed Vac (Savant Instruments Inc., Farmingdale, NY). The dried FQ aliquots were stored at -20 °C. The dried FQ was dissolved in running buffer to a final concentration of 5 mM on the day of the experiment and stored on ice.

Instrumentation

The CE instrument was built in-house and uses a post-column LIF detection as previously described (11). A 12 mW argon ion laser beam at 488 nm was used for excitation.

Labeling reaction and capillary electrophoresis

The protein/KCN solution was injected at 50 V/cm for 5 s. The capillary tip was washed twice with running buffer to minimize contamination. The FQ solution was electrokinetically injected at 50 V/cm for 5 s. The capillary tip was immersed in a vial of running buffer that had been heated to 65 °C in a dry bath incubator (Fisher Scientific, Edmonton, AB). After a 30 second reaction, the capillary was immersed in running buffer at room temperature. The CE separation was carried out at 400 V/cm. A 50 μm i.d. × 150 μm o.d. capillary with a length of either 35 or 40 cm was used. The running buffer was 2.5 mM borax and 5 mM SDS at pH 9.3.

Cell extract preparation and analysis

Phosphate buffered saline (PBS) solution was prepared by mixing 8 g NaCl, 0.2 g of KH_2PO_4, 0.46 g Na_2HPO_4, and 0.2 g of KCl and diluting to 1.00 L in distilled water. Roughly 10^6 HT29 human colon adenocarcinoma cells were washed five times with PBS and resuspended in 100 μL of water. The cells were sonicated for 20 minutes. The suspension was centrifuged at 1000 RPM (600 × G) for 10 minutes. Three microliters of the supernatant was mixed with 3 μL water and 2 μL of a 10 mM solution of NaCN.

A 250 pL plug of the extract was hydrodynamically injected at 11 kPa pressure for three seconds. A 10 mM solution of FQ was injected for 1 second. Next, the running buffer (50 mM phosphate, 11 mM SPS pH 6.8) was injected electrokinetically at 50 V/cm for 5s. The solution was incubated at 65 °C for 3 minutes and then separated at 400 V/cm.

RESULTS AND DISCUSSION

In EMMA, the reagents must mix in the capillary. FQ, the labeling reagent, is neutral and has an overall mobility equal to the electroosmotic mobility, which is roughly 6.7×10^{-4} $cm^2/V\text{-}s$. at pH 9.3. Both ovalbumin and cyanide are negatively charged at this pH and will have lower net mobility than FQ. We mix the protein and cyanide prior to analysis and inject them first onto the capillary; FQ is added in a second injection. In principle, CN^- and FQ could be mixed before injection; however, they react and form fluorescent impurities that interfere in the analysis of very low concentration proteins. Temperature has an important effect on sensitivity and reaction efficiency. The reaction rate was 15 times higher at 65 °C, the highest temperature used in the experiment, compared with room temperature.

We routinely labeled and analyzed proteins at low picomolar concentration at 65 °C. Figure 1 presents an electropherogram for a 5 pM conalbumin sample and a reagent blank. The blank peak-height was quite reproducible, with a 5% relative standard deviation.

A plot of peak height versus conalbumin concentration was linear ($X^2_\nu = 0.25$, $P = 0.55$ for 1 degree of freedom) from 0 pM to 10 pM in conalbumin concentration; the calibration curve was nonlinear at higher protein concentration, presumably because of a combination of peak broadening, ionic strength effects causing decreased stacking, and second-order reaction kinetics with the large number of lysine residues present at higher protein concentration. The assay detection limit (3σ) for ovalbumin was 3×10^{-13} M, which is 30 times superior to the detection limit reported for precolumn labeling at room temperature. It is important to note that these assay detection limits refer to the minimum amount of unlabeled protein required for analysis. Our assay detection limit is 7,000,000 times superior to the detection limit of 2×10^{-6} M produced by a commercial instrument with absorbance detection (11). Our assay detection limit is a factor of ten superior to the detection limit of 3×10^{-12} M reported for native fluorescence detection with stacking (1).

Protein mixtures can be labeled on-column and then separated by CE-LIF. A mixture of standard proteins was separated and detected by EMMA-CZE, Figure 2. This assay required five minutes: 30 seconds to inject the reagents, 30 seconds to perform the reaction, and 4 minutes to separate the products.

Real samples can be easily and efficiently analyzed by EMMA-CE. Figure 3 presents the electropherogram generated from a protein extract of an HT29 cell line (human colon adeno-carcinoma). Roughly a dozen major components and many minor components are resolved in the electropherogram in a 12 minute separation. This assay differed slightly from our earlier results in that hydrostatic pressure, rather than an electric field, was used to inject the sample/CN^- mixture and the FQ reagent. Pressure injection avoids the effects of differential mobility of the different materials. Once injected, the reagents are mixed by briefly applying an electric field to the capillary.

Other reagents have been used to analyze amino acids from single cells (21-22). To test if the peaks of figure 4 were due to proteins or amino acids, we mixed the protein extract with standard amino acids to generate a 10^{-7} M solution and repeated the experiment. None of the amino

acids generated peaks that were detected above the cell extract signal. FQ has relatively low reaction efficiency with amino acids compared with proteins. The reagent instead reacts much more efficiently with the ε-amine group of lysine residues on proteins (23).

The injection volume of 250 pL in the electropherogram of figure 3 contained the protein content of 2.5 cells. Given the excellent signal-to-noise ratio, it has not escaped our notice that this technique may be used to generate protein maps from single eukaryotic cells.

CREDIT

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FIGURE CAPTIONS

Figure 1. Electropherogram for 5 pM conalbumin and a reagent blank, labeled and analyzed using EMMA.

Figure 2. Separation of protein mixtures after EMMA labeling. The concentration listed in the figure corresponds to the concentration of unlabeled protein injected onto the capillary.

Figure 3. Analysis of a protein extract from an HT29 cell line.

Figure 1

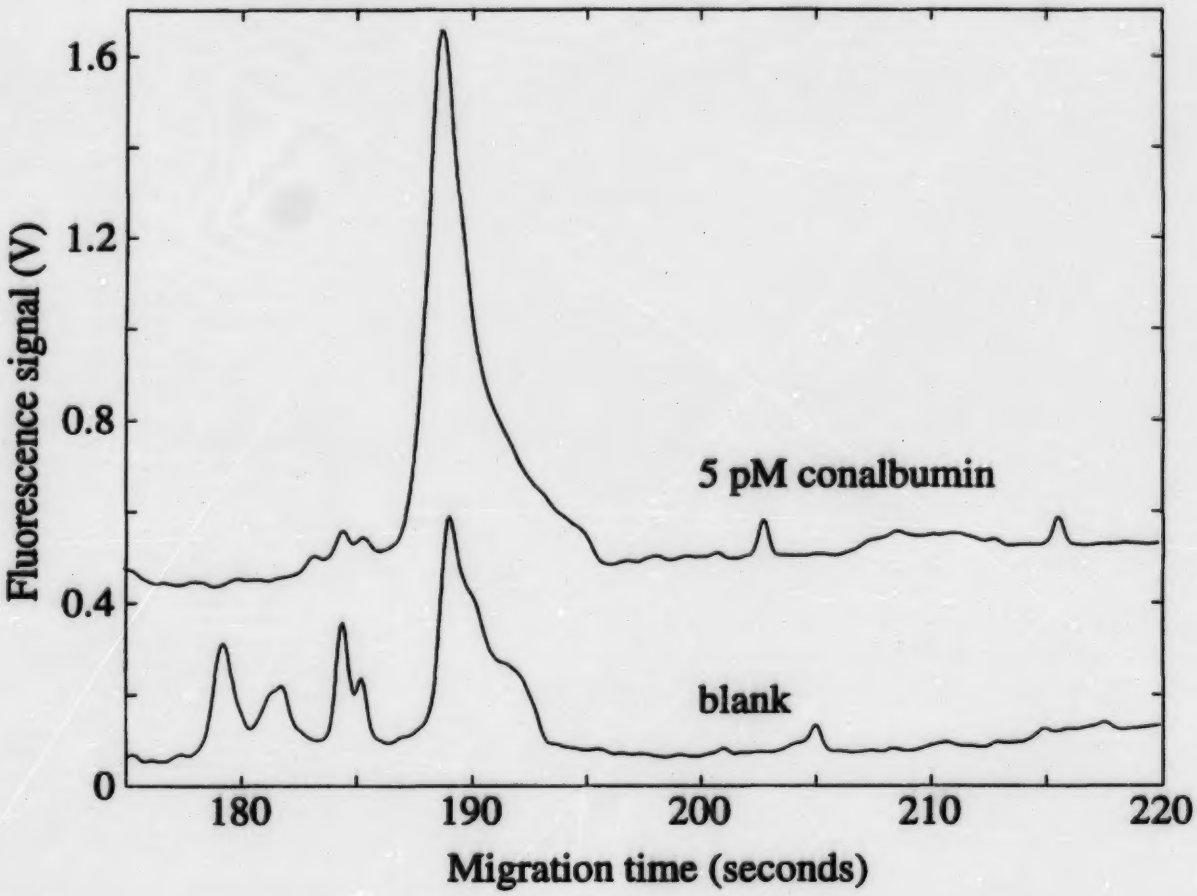

1.6
1.2
0.8
0.4
0
Fluorescence signal (V)
5 pM conalbumin
blank
180
190
200
210
220
Migration time (seconds)

Figure 2

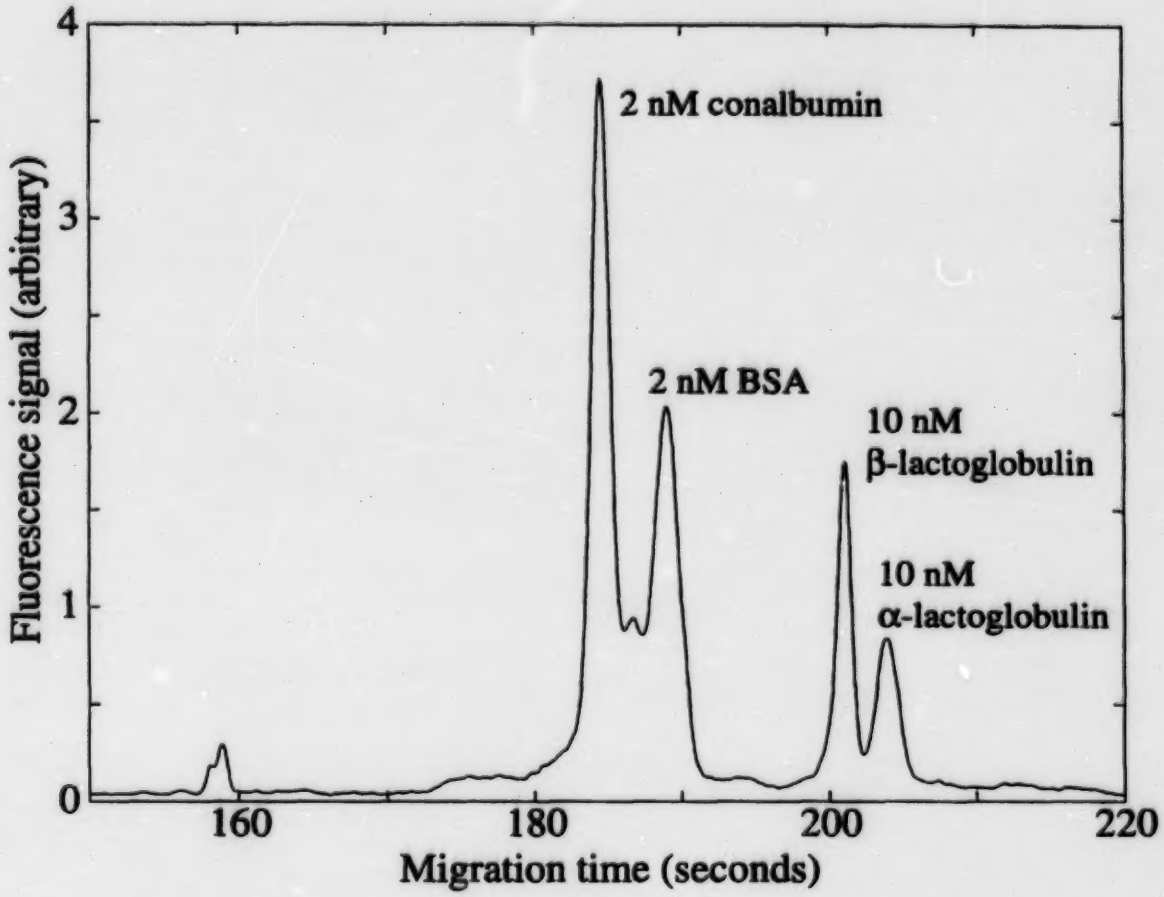
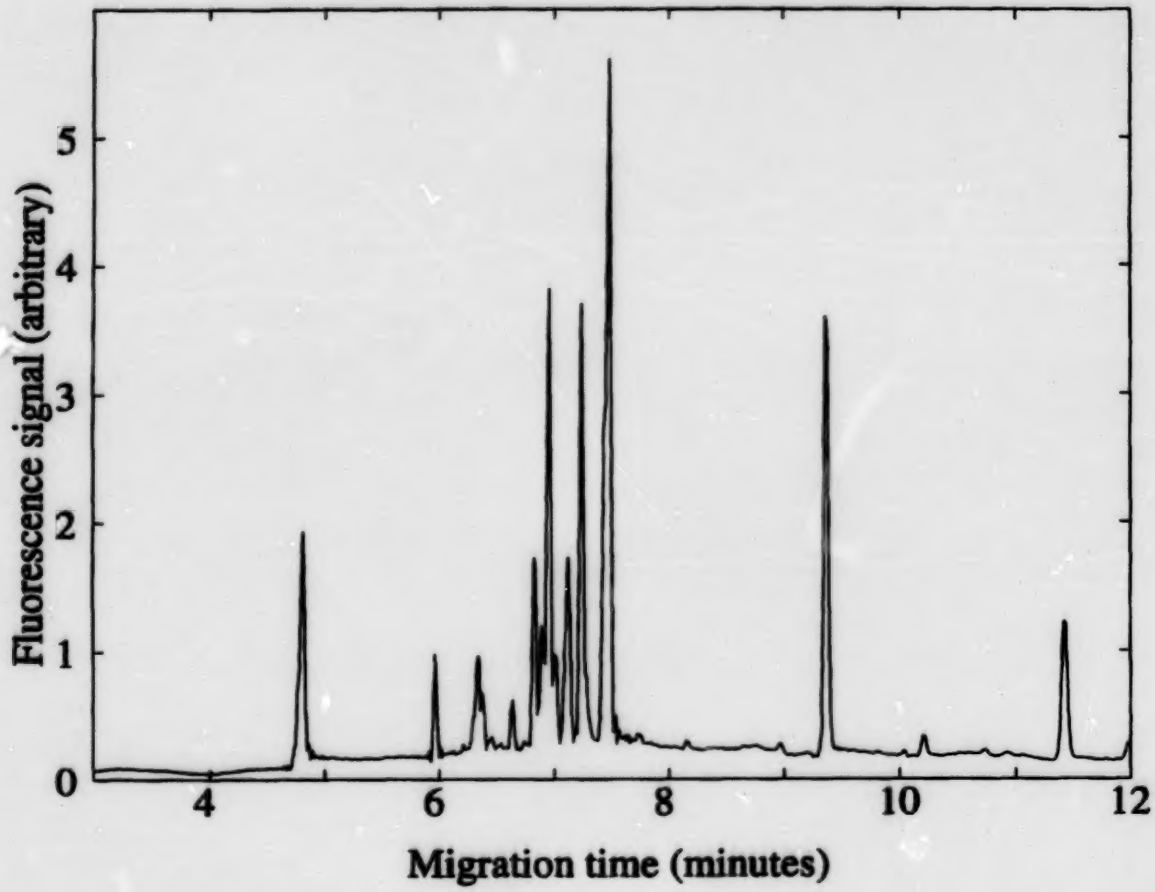

Fluorescence signal (arbitrary)
0
1
2
3
4
160
180
200
220
Migration time (seconds)
2 nM conalbumin
2 nM BSA
10 nM
β-lactoglobulin
10 nM
α-lactoglobulin

Figure 3


Fluorescence signal (arbitrary)
0
1
2
3
4
5
4
6
8
10
12
Migration time (minutes)